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26 **Metabolic and transcript analysis of the flavonoid pathway in diseased and recovered**
27 **Nebbiolo and Barbera grapevines (*Vitis vinifera* L.) following infection by Flavescence dorée**
28 **phytoplasma**

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44 **Running head : Flavonoid changes in phytoplasma infected vines**

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53 **ABSTRACT**

54 Flavescence dorée phytoplasma (FDp) infections seriously affect production and survival of
55 grapevine. We analysed the changes in the flavonoid pathway occurring in two red cultivars, the
56 highly susceptible Barbera and the less susceptible Nebbiolo, following FDp infection. A
57 combination of metabolic and transcript analyses was used to quantify flavonoid compounds and
58 expression of a set of genes involved in their biosynthesis. Quantification of anthocyanins,
59 flavonols, proanthocyanidins, and related biosynthetic enzymes, was performed over the vegetative
60 season, at four time points, on healthy, infected and recovered plants. A strong activation of
61 anthocyanin accumulation was observed in infected Barbera leaves, while the response was less
62 marked in Nebbiolo. Proanthocyanidins also accumulated mainly in infected Barbera leaves, even if
63 basal proanthocyanidin concentration was higher in healthy and recovered Nebbiolo. Biochemical
64 data were supported by transcript analysis: genes of the stem flavonoid pathway and of the
65 anthocyanin and proanthocyanidin branches were expressed at a higher level in infected than in
66 healthy plants, with a different magnitude between the two cultivars. Based on our results, we
67 hypothesize that flavonoid accumulation is a physiological consequence of FD infection without
68 affecting phytoplasma multiplication, although proanthocyanidin accumulation could help repel
69 further infection by the insect vector.

70

71 Keywords: Flavescence dorée; recovery; gene expression; anthocyanins; tannins; flavonols;
72 spectrophotometry; HPLC; quantitative PCR

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75 INTRODUCTION

76 Grapevine (*Vitis vinifera* L.) is cultivated worldwide and plays a pivotal role in the economy of
77 many countries, both for fresh consumption and wine production. Grapevine yield and quality can
78 significantly decrease because of biotic stress; beside well-known fungal pathogens and viruses,
79 recently phytoplasmas have become increasingly important as serious threats to vineyard survival in
80 several European grape growing areas. Taxonomically, phytoplasmas are plant-pathogenic bacteria
81 belonging to the class *Mollicutes*, a group of wall-less micro-organisms phylogenetically related to
82 low G+C, Gram-positive bacteria (Weisburg *et al.* 1989; Woese 1987). In the plant host they are
83 restricted to nutrient-rich phloem sieve tubes and are transmitted by phloem-sap feeding leafhoppers
84 or psyllids in a persistent propagative manner. Flavescence dorée phytoplasma (FDp, elm-yellows
85 group) and Bois Noir phytoplasma (stolbur group), are associated with important phytoplasma
86 diseases of grapevine (Osler *et al.* 1993). Due to its epidemic nature, FDp is a quarantine pathogen
87 in Europe. Use of certified phytoplasma-free propagation material, removal of infected plants from
88 the vineyard, and managing insect vectors are the only indirect approaches available to limit its
89 spread. Phytoplasmas are difficult to control and affect grapevine physiology dramatically. The
90 pathogen spreads through the plant tissues without inducing an effective resistance response;
91 infection is associated with alterations of physiological parameters (Bertamini & Nedunchezian
92 2001; Bertamini *et al.* 2002; Endeshaw *et al.* 2012; Rusjan *et al.* 2012a; Rusjan, Veberic &
93 Mikulic-Petkovsek 2012b) and of several metabolic pathways, as described at the molecular level
94 by qRT-PCR transcript quantification (Gambino *et al.* 2013; Hren *et al.* 2009b; Landi & Romanazzi
95 2011; Santi *et al.* 2013), microarray analysis of the transcriptome (Albertazzi *et al.* 2009; Hren *et al.*
96 2009a), and, very recently, proteomic analysis (Margaria, Abba & Palmano 2013; Margaria &
97 Palmano 2011). These physiological and molecular effects are linked to dramatic symptoms on
98 infected plants, such as reddening/yellowing of the leaves depending on the variety, downward
99 curling of leaves, stunting, lack of shoot lignification, and fruit abortion, finally resulting in severe
100 yield reduction at the end of the season, and even death of the vines.

101 Following initial infection and symptom expression, the disease can re-appear in the following
102 years, or disappear completely (recovery phenomenon). In the last case the recovered plant may
103 remain asymptomatic if not exposed again to infective vectors.

104 Flavonoids are a large class of plant secondary metabolites, encompassing more than 10,000
105 structures, that accumulate in fruit, flowers and leaves (Falcone Ferreyra, Rius & Casati 2012).
106 These molecules play several roles including regulation of plant cell physiology (Mahajan, Ahuja &
107 Yadav 2011), attraction of animal pollinators (Petroni & Tonelli 2011), response to abiotic (Berli
108 2010; Tohge *et al.* 2011) and biotic stress (Gutha *et al.* 2010; Miranda *et al.* 2007; Vega *et al.*
109 2011), and defence against herbivores and insects (Misra *et al.* 2010; Mohanta *et al.* 2012). In
110 grapevine, flavonoids are one of the most important determinants of quality due to their
111 involvement in determining fruit colour and taste. This importance is well documented by the vast
112 literature exploring flavonoid biosynthesis and accumulation in grape berries (Ali *et al.* 2011;
113 Azuma *et al.* 2012; Castellarin *et al.* 2011; Ferrandino *et al.* 2012). Previous studies explored the
114 alterations of flavonoid metabolism in grapevine during biotic stress, such as fungal (Rotter *et al.*
115 2009; Ali *et al.* 2012; Boubakri *et al.* 2013; Latouche *et al.* 2013) and virus infection (Gutha *et al.*
116 2010; Vega *et al.* 2011). According to phytoplasma infection, a few studies have described changes
117 in expression of some genes of the flavonoid pathway in grapevine leaves and berries during Bois
118 Noir phytoplasma infection (Albertazzi *et al.* 2009; Hren *et al.* 2009a; Landi & Romanazzi 2011;
119 Rusjan *et al.* 2012a; Rusjan *et al.* 2012b). However, so far, an integrated metabolomic and
120 transcriptomic approach has not been applied to study the impact of FD phytoplasma on grape
121 flavonoid biosynthesis and accumulation.

122 Here, we investigated the changes in the flavonoid pathway occurring in two important red grape
123 varieties from northern Italy, Nebbiolo and Barbera, base cultivars of some of the world's most
124 appreciated and valued red wines such as Barolo, Barbaresco and Barbera. Nebbiolo and Barbera
125 show different sensitivity to FD infection, the latter being more susceptible than the former. In fact,
126 FDp titre has been reported to be higher in Barbera than in Nebbiolo, which actually sustains a

127 lower multiplication of the pathogen during the season (Roggia *et al.* 2013). In the present study,
128 biochemical and transcript analyses were made for each cultivar over the 2011 season (four time
129 points) under three sanitary conditions (healthy, infected and recovered plants). Quantification of
130 total phenolics, anthocyanins and proanthocyanidins, followed by specific analysis of flavonols
131 were performed by spectrophotometry and HPLC-DAD, respectively. Key target genes involved in
132 the synthesis of these secondary metabolites, and relative regulatory elements, were quantified by
133 quantitative reverse-transcription (qRT)-PCR. The overall results showed temporal and variety-
134 specific patterns of compound accumulation and gene expression.
135 This is the first integrated biochemical and molecular analysis of several branches of the flavonoid
136 pathway occurring over-time in two grapevine cultivars with a different susceptibility to FD, under
137 two distinct physiological states: during active phytoplasma-induced infection and following
138 recovery from the disease.

139

140 **MATERIALS AND METHODS**

141 **Vineyards, plant collection and phytoplasma detection**

142 Two vineyards, located in Cocconato and Monteu Roero, Piemonte, in northern Italy, were used for
143 material collection in 2011. The choice of two different vineyards was necessary as our
144 experimental work was performed on healthy, phytoplasma-infected and stable recovered plants
145 (two years old recovery) of two varieties. Therefore we needed to sample from vineyards where the
146 sanitary situation was well known, in our case since 2007. The two vineyards have the same
147 exposure and altitude conditions (350m); vine spacing is 1.0 m x 1.2 m; rows are oriented North-
148 South; electronic meteorological stations of the Phytosanitary Service, Agrometeorological Sector,
149 Piemonte Region, were available for each vineyard and used to monitor climatic parameters. The
150 cultural practices adopted in the two vineyards were those traditionally managed by grape-growers
151 in the cultivation areas in Piemonte, following the requirements for the production of 'Controlled
152 Denomination of Origin' (DOC) wines. In particular neither irrigation nor chemical fertilization

153 were applied during the year; in both vineyards the grass between the rows was periodically
154 mowed, cut up and left on the ground. The vines were trained to the vertical shoot system and
155 Guyot pruned; before the vegetative season plants were mechanically and manually pruned, while at
156 the beginning of the season young shoots were mechanically pruned to ensure an homogenous vine
157 canopy architecture.

158 Four time points, spanning from June to September, were selected for the time-course experiment.
159 Collection dates were June 16, July 20, August 11 and September 8. Leaves from healthy and
160 infected plants were sampled at each date, whereas leaves from recovered plants were collected
161 starting from July. Plants sampled as healthy and recovered did not show any symptom of
162 phytoplasma infection, whereas infected plants showed typical symptoms (Margaria *et al.* 2007).
163 The leaves were collected for each plant from both sides of the row to attenuate the influence of
164 light exposure on certain classes of flavonoids, and flavonols in particular (Downey, Harvey &
165 Robinson, 2004). All sampled leaves were detached from the external vegetation layer between the
166 5th and the 8th node of the vegetative shoot, to ensure the collection of samples of the same age over
167 the season. We performed molecular assays on each single sampled plant to detect FDp (Margaria,
168 Turina & Palmano 2009; Margaria & Palmano, 2013), and exclude the presence of any other known
169 phytoplasmas in infected grapevines, as well any phytoplasma infection in healthy and recovered
170 plants. The phytoplasma strain was characterized (Martini *et al.* 1999) as belonging to subgroup
171 FD-C. All sampled plants were free of symptoms related to other known bacterial, fungal or viruses.
172 Finally, we selected for the experimental work 15 healthy plants, 15 FDp-infected plants and 12
173 recovered plants for each variety. All the recovered plants considered in this work were stably
174 recovered since two years.

175

176 **Experimental design**

177 For each sanitary state, we constituted three independent pools in each date (biological samples I,
178 II, III), each one composed by 5 different independent plants for the infected and healthy status and
179 by 4 independent plants for the recovery status. The plants considered and grouped at the first
180 sampling date, were then sampled in the same way at the following dates. In total we had 33
181 biological samples for each variety over the season, consisting in 12 healthy, 12 infected and 9
182 recovered biological samples, as shown in Supporting information Fig S1. From each pool, we used
183 10 g of plant material for metabolite analysis, which were stored at -20 °C in a pH 3.2 ethanolic
184 buffer (Ethanol 120mL, tartaric acid 5g/L, sodium bisulfate 2 g/L, neutralized with 3 vol of NaOH
185 1N) until analysis. At the same time, 1 g of each pool was frozen in liquid nitrogen and kept at -80°
186 C until RNA extraction. Another aliquot of 1 g of the FD positive samples, was stored at -80° C and
187 used for DNA extraction and phytoplasma titer quantification (Roggia *et al.* 2013).

188

189 **Extraction and quantification of total phenolics, total anthocyanins and total** 190 **proanthocyanidins**

191 Leaves were thawed with an Ultraturrax dispersing machine (IKA, Staufen, Germany), equipped
192 with a dispersing element, 22 cm of length and 1.2 cm of diameter and centrifuged at 4000 rpm.
193 The supernatant was stored; the pellet was re-suspended and centrifuged again; the supernatant
194 from the second centrifugation was added to the first supernatant and the mixture was brought to a
195 final volume of 100 ml. Samples were stored at -20 °C until analysis.

196 For determining the total phenolics and total anthocyanin content, leaf extracts were diluted (from
197 12.5 to 25 fold, depending on the expected anthocyanin concentration of samples) with ethanol
198 chloride (CH₃CH₂OH: H₂O: HCl, 70:30:1) (Di Stefano, Cravero & Gentilini 1989).

199 Spectrophotometric indexes of total phenolics, with major component of flavonoids (absorbance
200 read at 280 nm) and total anthocyanins (absorbance read at 520 nm) were calculated and quantified
201 using (+)-catechin hydrate (Fluka) and malvidin 3-O-glucoside chloride (Extrasynthèse, Genay
202 Cedex, France) as external standard reference, respectively. Total phenolics were expressed as

grams of (+)-catechin hydrate equivalents per kg of fresh leaf with ϵ at 280 nm of $3487 \text{ L mol}^{-1} \text{ cm}^{-1}$ for (+)-catechin hydrate dissolved in ethanol chloride. In order to avoid possible interferences by SO_2 -rich solvent [38] at 280 nm spectrophotometric measures, a blank was used containing the extractant SO_2 rich buffer and chloridric ethanol in the same concentration as the samples. Total anthocyanins data were expressed as grams of malvidin 3-O-glucoside chloride equivalents per kg of fresh leaf; the molar extinction coefficient (ϵ) at 542 nm of for malvidin 3-O-glucoside chloride dissolved in ethanol chloride was $29997 \text{ L mol}^{-1} \text{ cm}^{-1}$. Total proanthocyanidins (PA) were measured by the protein precipitation method (Harbertson, Kennedy & Adams, 2002), adapted to analyze amounts of PAs in grape leaf extracts. A protein solution (bovine serum albumin, BSA) with a final concentration of 1 mg/mL was prepared in a pH 4.9 buffer (200 mM acetic acid and 170 mM NaCl); a 1 mL aliquot of the protein solution was dispensed into a 1.5 mL microfuge tube, then $500 \mu\text{L}$ of the extract of grape leaves were added and the mixture was incubated at room temperature for 15 min with slow agitation. After incubation the sample was centrifuged for 5 min at 13500 g to pellet the PA-protein precipitate. The liquid solution was poured off and the pellet was washed with $250 \mu\text{L}$ of the same buffer used to dissolve the BSA (200 mM acetic acid and 170 mM NaCl adjusted to pH 4.9 with NaOH). Sample was centrifuged again to re-pellet the PA-protein precipitate. The wash solution was discarded, and $875 \mu\text{L}$ of a buffer containing 5% of triethanol amine (TEA, v/v) and 10% of lauryl sulphate sodium salt (SDS, w/v) were added, and the tube was allowed to stand at room temperature for 10 min . The tube was then vortexed to completely dissolve the PA-protein pellet. The solution was allowed to stand at room temperature for 10 min and the background absorbance at 510 nm was read. Subsequently, $125 \mu\text{L}$ of a ferric chloride reagent (10 mM FeCl_3 in HCl 0.01 N) was added, and after 10 min , the absorbance at 510 nm was recorded again. The absorbance was determined by subtracting the background absorbance from the final reading at 510 nm . A zero epicatechin sample was prepared by adding $125 \mu\text{L}$ of the FeCl_3 reagent to $1.875 \mu\text{L}$ of the TEA/SDS buffer and absorbance at 510 nm of this solution was subtracted from each of the points on the standard curve. PA concentration in leaves were expressed

229 as grams of (-)-epicatechin gallate equivalents per kg of fresh tissues. A standard curve was
230 prepared using (-)-epicatechin gallate in the range of 25 to 500 mg/L.

231

232 **Extraction and quantification of individual flavonols**

233 Leaf extracts were diluted 1.1-fold with phosphoric acid 1 M; flavonols were separated and detected
234 by HPLC/DAD (Perkin Elmer series 200-L pump) equipped with a Licrosphere 100 RP-18 5 mm
235 column (25 x 0.4 cm ID) (Merck, Darmstadt, Germany). The chromatographic method, peak
236 identification and quantification were performed accordingly to a previous published method
237 (Ferrandino & Guidoni, 2010). Total flavonols were expressed as milligrams of quercetin 3-O-
238 glucoside equivalents per kilogram of fresh leaves. The percentages of individual flavonols were
239 also calculated.

240

241 **RNA extraction from grapevine leaves**

242 Total RNA from *V. vinifera* leaves was extracted following a published protocol (Reid *et al.* 2006)
243 with some modifications. One g of leaf tissues was ground in a mortar with liquid nitrogen and
244 immediately transferred to 50 mL tubes containing 20 mL of pre-warmed (65°C) extraction buffer
245 [2% hexadecyltrimethylammonium bromide (CTAB), 2% polivinilpirrolidone 40 (PVP40), 10 mM
246 Tris-HCl pH8, 25 mM EDTA, 2M NaCl, 2% β -mercaptoethanol), vortexed and incubated for 10
247 min at 65°C. Tubes were added of 1 vol chlorophorm-isoamilic alcohol 24:1, vortexed for 2 min
248 and centrifuged at 3000 g for 30 min at 4°C. Supernatant was transferred to a new tube. One
249 volume of chlorophorm-isoamilic alcohol 24:1 was added, vortexed and centrifuged at 3000 g for
250 45 min. The supernatant was further centrifuged at 30,000g for 10min at 4°C, and next added with
251 0.1 volume 3M NaOAC (pH 5.2) and 0.6 volume isopropanol. Following incubation at -80°C for 1
252 h, tubes were centrifuged at 3500 g for 30min at 4°C. Pellet was dissolved in 1mL of 1X TE
253 pH7.5; 0.3 volume of LiCl 9 M was added and left overnight at 4°C. RNA was precipitated by
254 centrifugation at 20,000 g for 30min at 4°C, washed with 70% cold ethanol and resuspended in 200

255 μ L of 0.1% DEPC-treated sterile water. RNA samples were treated with RNase-free DNase I
256 (Applied Biosystems, Foster City, CA, USA) in the supplied buffer to avoid residual DNA
257 contamination: 1 μ g was digested for 1h at 37°C with 2 DNase units in 40 μ L final volume. DNase
258 was removed by phenol/chloroform extraction following the manufacturer's instructions. RNA was
259 resuspended in 30 μ L of RNase-free water treated with 0.1% DEPC, and quality was analysed by a
260 spectrophotometer to evaluate integrity, purity and concentration of the extracts. The RNA aliquots
261 were stored at -80 °C.

262

263 **Selection of target and reference genes**

264 Thirteen genes were considered in this study. Eight target genes coded for key enzymes of the
265 flavonoid pathway: chalcone synthase isoforms II (*CHS II*, GSVIVT01032968001) and III (*CHSIII*,
266 GSVIVT01000521001), flavanone-3-hydroxylase 2 (*F3H2*, GSVIVT00014419001),
267 leucoanthocyanidin dioxygenase (*LDOX*, GSVIVT00001063001), UGT-glucose:anthocyanin 3-
268 Oglucosyltransferase (*UAGT*, GSVIVT00014047001), flavonol synthase 1 (*FLS*,
269 GSVIVT00015343001), anthocyanidine reductase (*ANR*, GSVIVT00005344001), and leuco-
270 anthocyanidine reductase (*LAR*, GSVIVT00030282001). Two CHS isogenes (*CHS II* and *CHSIII*)
271 were considered, in order to monitor for possible changes in the contribution of each isoform to the
272 pathway, as observed previously in the Grapevine-leafroll-associated virus-3 (GLRaV-3) interaction
273 (Zabala *et al.* 2006). Further reactions involving *F3H*, *LDOX* and *UAGT* enzymes lead to the
274 synthesis of anthocyanidin pigments. Other branches include *FLS*, for the synthesis of flavonols,
275 and *LAR* and *ANR* for synthesis of flavan-3-ols, the precursors of proanthocyanidin (PA) polymers.
276 In a few cases, a specific gene isoform was selected on the basis of previous literature reports
277 showing significant changes during biotic infection in grapevine.

278 Two genes coding for transcription factors were also chosen as targets: *VvMYBA1*
279 (GSVIVT01022659001), which is involved in regulation of *UAGT* and anthocyanin biosynthesis

280 (Cutanda-Perez *et al.* 2009), and *VvMYBF1* (GSVIVT00028082001), which regulates flavonol
281 synthesis (Czemmel *et al.* 2009).

282 Three endogenous reference genes, actin (*ACT*, GSVIVT00034893001), ubiquitin (*UBI*,
283 GSVIVT01038617001) and ribosomal gene *18S* were used for evaluation of the stability of gene
284 expression in our conditions.

285 All primer pairs used in this study were derived from the literature and references are included in
286 Supporting Information Table S1, together with gene accession number and genome based gene
287 identifier. In order to exclude differences in the gene sequences between the two genotypes, that
288 could negatively impair the ability of the primers to amplify the target DNA, the efficiency of
289 amplification was determined for each gene in each variety, by construction of dilution curves, as
290 described in the following paragraph.

291

292 **Real-time qRT-PCR assays**

293 For qRT-PCR, first-strand cDNA synthesis was made in duplicate in two independent runs using
294 100 ng of total DNase treated RNA and random examers as primers, according to the High Capacity
295 cDNA Reverse Transcription kit (Applied Biosystems). Two µL of cDNA were used as template in
296 the qPCR reactions, containing 12.5 µL of IQTM Supermix (Bio-Rad, Life Science Research,
297 Hercules, CA, USA) and 200 nM primers (Supporting Information Table S1), to a final volume of
298 25 µL. Reaction conditions for all primer pairs were as follows: 3 min at 95 °C, 40 cycles of 15 s at
299 95 °C, 10 sec at 56 °C and 30 sec at 72°C. At the end of each qPCR, melting curve analysis was
300 performed over the range 56-95°C to determine the specificity of amplicons. Reactions were carried
301 out in the StepOne Plus RealTime PCR System (Applied Biosystems).

302 The sample maximization approach (Derveaux, Vandesompele & Hellemans 2010) was used for
303 plate design, i.e. all samples for each variety (33 total) were simultaneously analyzed in the same
304 plate for each target gene, in duplicate. In order to estimate qPCR efficiency, a standard curve
305 consisting of at least four 1:5 fold dilution points of the cDNA obtained from a Master sample was

306 included in each plate; preliminary assays were performed in order to evaluate the optimal dilutions
307 for each gene target. The Master sample was prepared by mixing all the 33 DNase-digested RNAs
308 of each variety, divided in aliquots stored at -80°C, and used for cDNA synthesis and standard
309 curve points in all the plates, except those used for *FLS* and *VvMYBF1* target genes. The low
310 concentration of these transcripts in the analyzed samples, in fact, hampered the construction of a
311 dilution curve from cDNA. Thus, for *FLS* and *VvMYBF1* RNAs quantification, standard curves
312 were prepared by using plasmids containing the fragment of interest. Amplified fragments
313 according to Gutha *et al.* 2010 and Czemmel *et al.* 2009, were purified and cloned in the pGEM-T
314 vector (Promega). Liquid cultures were set up and plasmids purified and sequenced. Ten-fold
315 dilutions starting from 60 fg/μL were used for determination of standard curves in qPCR reactions.
316 Standard curves were derived by linear regression analysis from plot of the threshold cycle values
317 of each standard dilution point against the Log of the arbitrary concentration of the DNA dilutions.
318 In order to evaluate the stability of the cDNA used in serial plate runs, two dilutions (1:5; 1:125) of
319 the Master sample were used to amplify the actin gene in all plates and Ct values were analyzed to
320 exclude cDNA degradation in serial qPCR reactions. In each plate, we included sterile distilled
321 water as template instead of RNA as no-template control, and a sample of RNA from the master
322 sample to monitor for possible DNA residuals in the samples.

323 324 **Validation of reference genes and relative gene expression analysis**

325 The stability of reference genes was determined by using three software tools commonly used for
326 reference gene selection: geNorm (Vandesompele *et al.* 2002), Norm Finder (Andersen, Jensen &
327 Orntoft 2004), and Best Keeper (Pfaffl *et al.* 2004) (Supporting Information Table S2). As input,
328 the mean of the Ct values of each sample reported as ΔC_t value to the Ct of the lowest concentrated
329 sample and corrected for PCR efficiency was used. The weighted mean of the expression ratios of
330 the two best genes was used as the normalization factor for relative quantities (RQ) calculation
331 according to the $2^{-\Delta\Delta C_t}$ method.

For each gene, the mean Ct value of healthy samples was taken as reference to calculate the relative expression levels of each sample, in each cultivar. $\Delta\Delta Ct_{weighted}$ was calculated, according to (Yuan, Wang & Stewart, 2008), using the PCR amplification efficiency (PAE) to adjust the $\Delta\Delta Ct$ values of each gene. As weight we used $w=0.5$, since we used two reference genes, RG1 and RG2 ($w=1$ divided by the number of reference genes). The resulting equation for calculating $\Delta\Delta Ct_{weighted}$ of each sample i for each target gene j was:

$$\Delta\Delta Ct_{weighted,j,i} = [Ct_{j,i} * PAE_j - (Ct_{RG1i} * PAE_{RG1} * w + Ct_{RG2i} * PAE_{RG2} * w)] + \\ - [meanCt_{j,h} * PAE_j - (meanCt_{RG1h} * PAE_{RG1} * w + meanCt_{RG2h} * PAE_{RG2} * w)]$$

where the suffix _h indicates the total healthy plants of the four sampling dates.

Relative quantities values ($RQ_{j,i} = 2^{-\Delta\Delta Ct_{weighted,j,i}}$) were calculated for each sample. From these, the mean relative quantity and its standard deviation was calculated for the three biological replicates of each sanitary status and of each sampling date, and used for further comparisons.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were separated by the Duncan's test at $P \leq 0.05$ and ≤ 0.01 with SAS 8.2 for Windows (SAS Institute, Cary, NC, USA).

Multiple regression analysis for correlating metabolites and gene transcripts was done using the software GenStat rel. 15 (VSN International, Hemel Hempstead, U.K.).

RESULTS

Symptoms and FDp quantification in the two cultivars

Symptoms severity varied between the two cultivars, with Barbera vines generally showing more severe symptoms, similar to what was observed in surveys carried out in previous years in the same vineyards (Roggia *et al.* 2013). Starting from the first sampling date, differences were observed: Nebbiolo was characterized by leaf downward rolling, withering of the inflorescence and mild yellows around the veins, while infected Barbera plants showed marked reduced growth of new

shoots, withering of the inflorescence, short internodes and leaf reddening. In late summer, Nebbiolo plants showed lack of shoot lignification, with reddening around veins and limited to leaf sectors; Barbera plants showed lack of shoot lignification, evident brown pustules on canes and marked reddening of the whole leaf often resulting in the whole plant turning purple (Fig. 1). No symptoms were observed on healthy and recovered plants, the last being classified as infected in the previous years. Each single plant was tested for phytoplasma infection, and FDp was found only in the grapevines showing typical symptoms, excluding the presence of any other phytoplasma in all samples. FDp titre was quantified in the two cultivars: mean FDp concentrations in infected Barbera samples were one order of magnitude higher than in infected Nebbiolo samples (Fig. 2a). A seasonal trend in FDp concentration was observed with peak in July for Nebbiolo and in August for Barbera, followed by a decrease during the rest of the season. Amplification was never observed neither in healthy nor in the recovered plants.

Regarding the climatic parameters, average temperatures in the two vineyards were very similar all over the year (Supporting Information Fig. S2), and especially during summer, when temperatures reached 20.4, 21.7, 24, 21 °C in Monteu Roero and 20.4, 21.4, 24.1, 21.2 °C in Cocconato, spanning from June to September. The growing degree days base 10 °C were 2210 in Cocconato and 2123 in Monteu Roero. The relative humidity during the year was 66.8 % in Cocconato and 73.7 % in Monteu Roero), with specific values of 79, 72, 65, 71% in Monteu Roero and 74, 68, 61, 69 % in Cocconato, from June to September.

377

378 **Total phenolics**

Total phenolic concentrations did not show differences between the three sanitary states (healthy, infected and recovered), until the second sampling (July) when infected vines accumulated more phenolics compared to healthy ones, in both varieties. In general, recovery did not affect the phenolic accumulation (Fig. 2b). The seasonal phenolic concentration increase in infected leaves compared to healthy ones, was higher in Barbera than in Nebbiolo; also the timing of the response

384 to FD infection was different between the two varieties. In fact, in Barbera (Fig. 2b), a peak was
385 detected at the third sampling, when phenolic concentration in leaves from infected vines was
386 almost double compared to that of healthy vines (+ 95 %), whilst, in Nebbiolo (Fig. 2b), the
387 phenolic maximum concentration was concomitant with the second sampling (+ 45 % in infected vs
388 healthy leaves).
389 In general, the trend of phenolic accumulation (Fig. 2b) reflected that of the phytoplasma titre
390 during the season (Fig. 2a) in both cultivars.

391

392 **Total anthocyanins, proanthocyanidins, and flavonols**

393 As leaf reddening is a typical symptom of FD in red-coloured grape varieties, we measured the
394 concentration of different classes of flavonoids, including anthocyanins, flavonols, and
395 proanthocyanidins in healthy, FDp-infected and recovered plants.

396 Anthocyanin concentration in leaves of both healthy and recovered plants was low and overall
397 similar in both varieties during the season, in agreement with lack of observable reddening. In
398 infected Barbera leaves, the reddening was visible since the first sampling time, when anthocyanin
399 accumulation was already statistically significant. The peak of anthocyanin accumulation was
400 detected at the third sampling, in coincidence with berry full véraison, and the concentration was up
401 to 10 times higher in infected leaves compared to healthy ones (Fig. 3a; Supporting Information
402 Table S3). In infected Nebbiolo, anthocyanin concentration was limited, likely reflecting the
403 localized and patchy redness generally observed in symptomatic leaves, and the concentration was
404 not more than twice that of healthy leaves (Fig. 3a; Supporting Information Table S3).
405 Nebbiolo grapes tend to accumulate more proanthocyanidins (PA) than Barbera, and this was also
406 evident in leaves, where basal PA concentrations (at first sampling) in healthy and recovered plants,
407 were higher in Nebbiolo. Proanthocyanidin concentration increased in infected samples of both
408 varieties, but with less evidence in Nebbiolo than in Barbera (Fig. 3b; Supporting Information Table
409 S4).

410 No difference in total flavonol concentration was detected between the three sanitary conditions in
411 both cultivars (Fig. 3c). In healthy leaves of both genotypes, the main flavonol aglycone was
412 quercetin, followed by kaempferol and myricetin, as reported in leaves of other *Vitis vinifera*
413 varieties (Fernandes *et al.* 2013), and this profile did not change in recovered and infected plants.
414 Neither in Nebbiolo nor in Barbera leaves differences were detected in the percentage concentration
415 of individual flavonols, irrespective of their sanitary status (Supporting Information Table S5).
416 However, some differences were detected between the two genotypes: Nebbiolo leaves were
417 characterized by a higher percentage of quercetin glycosides compared to Barbera, and lower
418 percentages of kaempferol and myricetin glucoside.

419

420 **Selection of reference genes for qRT-PCR analysis and primer validation**

421 The expression pattern of eight biosynthetic and two regulatory genes was monitored in grapevine
422 leaves collected from healthy, recovered, and infected plants. As the selection of appropriate
423 reference genes is critical for qRT-PCR data analysis, and considering the complexity of our
424 experimental design (two cultivars, three sanitary conditions, four time points), we decided to
425 monitor the stability of expression of three reference genes (actin, ubiquitin and 18S). Three
426 statistical tools were used, i.e. Best Keeper, geNorm and Norm Finder. Output values from the three
427 softwares for each reference gene are reported in Supporting information Table S2. In Barbera,
428 actin was selected as the best reference gene by all programs. In Nebbiolo, geNorm selected actin,
429 while Norm Finder and Best Keeper selected the ubiquitin gene, although also actin was highly
430 stable. In this study, we finally decided to use the weighted mean of the expression ratios of actin
431 and ubiquitin as the normalization factor for relative quantities (RQ) calculation in both varieties.
432 All primers used in the study showed very similar amplification efficiency and very high R^2
433 values in Barbera and Nebbiolo (Supporting information Table S1), and were then used in the Real-
434 Time RT-PCR assays as described.

435

436 **Expression patterns of flavonoid biosynthetic genes**

437 *Gene expression in Barbera leaves*

438 Early genes of flavonoid biosynthesis (*CHS* and *F3H*) contribute to the production of
439 dihydroflavonols, which are then further processed to flavonols, anthocyanins and
440 proanthocyanidins. In Barbera, a marked difference in the expression of two *CHS* isogenes was
441 observed (Fig. 4): RQs of *CHS3* showed a large increase in infected leaves, with a peak in August
442 followed by decrease afterwards. A similar profile was not observed for *CHS2* which, moreover,
443 showed high variability among biological replicates. *F3H2* mRNA concentration levels in infected
444 tissues also increased during the season, reaching RQ values up to 7 times those observed in healthy
445 samples in August, followed by a decrease (Fig. 4).

446 An expression trend similar as for the early biosynthetic genes *CHS3* and *F3H2* was observed for
447 the *LDOX* and *UAGT* genes, which are addressed to anthocyanin biosynthesis (Fig. 5). As expected,
448 based on the anthocyanin concentration data, expression of these genes in infected Barbera plants
449 showed an increase of 35 and 170 fold respectively above healthy plants, at the peak of expression.
450 Accordingly, the expression of the *UAGT*-transcription factor *VvMYBA1* (Fig. 5), was significantly
451 higher in infected plants in July (about 80 times the healthy samples), and later decreased.

452 *LAR* and *ANR* are involved in proanthocyanidin synthesis, respectively producing catechin and epi-
453 catechin from di-hydroxylated substrates. No significant differences in the RQ values were
454 observed for the single-copy gene *ANR* (Fig. 6). In grapevine, *LAR* is present in two isoforms, *LAR1*
455 being more expressed in leaves (Bogs *et al.* 2005). *LAR1* transcripts showed increased accumulation
456 during the season in phytoplasma-infected leaves, starting in August till the last sampling point
457 (Fig. 6). The flavonol branch of the flavonoid biosynthetic pathway was probed analysing
458 expression of the enzyme-coding gene *FLS1*, and of the cognate transcription factor *VvMYBF1*.
459 Both failed to show significant changes among the four time points and, accordingly, no differences
460 were observed among the sanitary states (Fig. 7).

461 Gene expression trends in recovered plants were similar to healthy plants and not significantly
462 different among sampling dates (Fig. 4,5,6,7).

463

464 *Gene expression in Nebbiolo leaves*

465 Differential expression of flavonoid biosynthetic genes in infected plants during the season was also
466 observed in Nebbiolo plants. As observed in Barbera, *CHS3*, *F3H2*, *LDOX*, *UAGT* and *VvMYBA1*
467 were up-regulated in infected plants (Fig. 4, 5), but their RQ values at the peak of expression were
468 markedly lower than in Barbera: about 5, 4, 7, 44, and 25 times than healthy samples. The first three
469 of the above mentioned genes showed the peak of expression in July, and no further transcript
470 accumulation was observed later in the season.

471 Within the proanthocyanidin pathway, *ANR* did not show increased expression in infected samples,
472 while *LAR1* reached the peak in July, with RQ value about 2 times those observed in healthy
473 samples (Fig. 6). No significant differences were observed for the expression of genes of the
474 flavonol branch, *FLS* and *VvMYBF1* (Fig. 7). Also in Nebbiolo, no differences were observed
475 between RQ values of healthy and recovered plants (Fig. 4,5,6,7).

476

477 **Correlations between metabolite concentration and transcript abundance**

478 *Relationships between anthocyanins and UAGT transcripts*

479 Significant correlations were found between the Log-transformed values of *UAGT* RQ and the Log-
480 transformed concentrations (g/kg) of anthocyanins, in infected samples for both the Barbera and
481 Nebbiolo vines ($\text{Log_Anthocyanins} = 0.4556 \text{ Log_RQ} - 0.6662$ for infected Barbera and
482 $\text{Log_Anthocyanins} = 0.1761 \text{ Log_RQ} - 0.57322$ for infected Nebbiolo; percentage variance
483 accounted for: 97.4; F probability of regression < 0.001). Healthy and recovered plants of both
484 cultivars, at all sampling dates, had levels of transcripts and anthocyanins so close to 0 that it was
485 not possible to correlate the two variables. In infected plants, the anthocyanin concentration was
486 related to transcript quantities in both cvs. As shown in Figure 8, the line slope was higher in

487 Barbera than Nebbiolo, indicating that in presence of equal *UAGT* RQ values, the repercussion of
488 transcript accumulation on anthocyanins concentration is stronger, in particular double, in Barbera.
489

490 ***Relationships between proanthocyanidins and LAR and ANR transcripts***

491 We have studied the relationships of the concentration of proanthocyanidins [PA] with the RQ of
492 both *LAR* and *ANR* transcripts for the two pooled cultivars. The model considered is:

493
$$[PA] = a * \text{Log_RQ_LAR} + b * \text{Log_RQ_ANR} + c.$$

494 The fitted models expressing the PA concentration in relation to *LAR* and *ANR* RQ was:

495
$$[PA] = 0.3888 * \text{Log_RQ_LAR} - 0.675 * \text{Log_RQ_ANR} + 0.9168$$
 (% variance accounted for 36.3 ; F
496 probability of regression < 0.001). No significant changes to the regression are introduced
497 separating the values according either to the cultivar or to the sanitary state (Fig. 9a).

498 Figure 9b show the distribution of the three sanitary states separately for Barbera and Nebbiolo for
499 an easier visualization. In Barbera, it is evident the clustering of infected samples toward the high
500 values of PA; instead, in Nebbiolo the single values of different sanitary states are not evidently
501 grouped in levels of PA.

502

503 ***Relationships between flavonols and FLS transcripts***

504 No significant correlation was found between *FLS* RQ and flavonols concentration. In fact FD
505 infection did not affected flavonol accumulation (Fig. 3c), neither the expression of the related
506 biosynthetic gene (Fig. 7).

507

508 **DISCUSSION**

509 This study is the first analysis of changes in different branches of the flavonoid biosynthetic
510 pathway, in FDp-infected, recovered, and non-infected grapevine plants, based on an integrated
511 metabolic and transcriptomic approach. Barbera and Nebbiolo are two traditional and economically
512 important Italian grapevine cultivars. The differential susceptibility of the two cultivars to FDp

infection is well known: Barbera is highly susceptible to FDp and shows severe symptoms, already visible in early summer, while symptoms on Nebbiolo vines are milder and not so evident until middle summer (Morone *et al.* 2007). Two vineyards in Piedmont were considered for sampling; this was necessary because we needed to sample from fields where the sanitary situation was known since many years, in order to sample stable recovered plants, i.e. plants that were recovered since 2 years. The symptomatology described for the two cultivars in the two vineyards reflected the situation which is commonly observed anywhere Barbera and Nebbiolo are planted and Flavescence dorée infection insists, with Barbera always more sensitive than Nebbiolo (Morone *et al.* 2007; Roggia *et al.* 2013). Moreover, the vineyards were located in a specific growing location which is representative of the denomination of origin area (Cocconato for Barbera d'Asti DOCG and Monforte Roero for Nebbiolo d'Alba) and where the cultural practices are the traditional ones requested for the production of 'Controlled Denomination of Origin' (DOC) wines, thus reflecting the real situation we can find in Piemonte with field-grown material. Even though we are aware of the important effects exerted by climatic conditions on grape quality (Ferrandino & Lovisolo 2013; Jakoola & Hohna 2010; Tossi *et al.* 2012), the main climatic parameters (temperature and relative humidity) were very similar. It has also been shown that light intensity and type (high or low solar UV-B) influenced the antioxidant capacity of leaves (Berli *et al.* 2013). However, it is known that the cultural practice management in the vineyard can be used to regulate the vine canopy, and thus the leaf exposure to light. In the two vineyards the cultural practices adopted were the same (see Materials and Methods for detail), so the influence exerted by the type of canopy management on the light interception (and, consequently, on the accumulation of secondary metabolites) could reasonably be excluded. As previously stated, biological observation based on symptom severity, indicated Barbera as a more susceptible cultivar to FDp infection than Nebbiolo; a recent study has shown that the two cultivars also support different phytoplasma titres. In particular, FDp concentration in Barbera was always higher than in Nebbiolo in two successive years, although no linear correlation between FDp titre and symptoms severity could be assessed (Roggia *et al.* 2013).

539 The phytoplasma titre in our samples confirmed the higher concentration in Barbera than Nebbiolo.
540 Absolute values (Fig. 2a) are comparable to those assessed in the report of Roggia *et al.* 2013, when
541 it is taken into account the variability in phytoplasma titres among years and that, in this study, we
542 extracted whole leaf samples, instead of using only veins, whose weight amounts to 5-10% of total
543 leaf tissue, consequently resulting in a diluted concentration of the phloematic pathogen. The
544 erratic phytoplasma distribution in woody hosts (Berges, Rott & Seemuller 2000), may have also
545 played a role in the quantitation results, as during the sampling we took material representative of
546 the whole plant without focusing just on symptomatic branches, commonly used for diagnosis.
547 Expression of flavonoid biosynthetic genes and accumulation of flavonoids is a common hallmark
548 of pathogen infection of plant leaves. Flavonoid biosynthesis in grapevine requires the stepwise
549 action of “stem” biosynthetic genes, which produce precursors used for the synthesis of specific
550 flavonoids in “branch” reactions. Within the “stem” biosynthetic genes, we analyzed expression of
551 two isoforms of *CHS* (*CHS2* and *CHS3*) and of *F3H2*.
552 Three *CHS* genes were described in grape: *CHS1* and *CHS2* are expressed in leaves and unripe
553 berries, while *CHS3* was reported to be limited to fruits (Goto-Yamamoto *et al.* 2002) and its
554 expression was correlated with anthocyanin accumulation in berry skins (Ageorges *et al.* 2006). In
555 our leaf samples, however, no significant accumulation of *CHS2* transcripts took place in infected
556 leaves, while *CHS3* expression markedly increased. This suggests that *CHS3* is strictly linked to
557 anthocyanin accumulation also in leaves, and that the other two *CHS* isoforms may be required for
558 accumulation of other types of flavonoids. This could be in agreement with the marked difference
559 in the magnitude of *CHS3* activation between the two varieties (Fig. 4), with a strong increase in
560 Barbera (800 times the healthy at the mean peak) with respect to Nebbiolo (5 times compared to
561 healthy plants) likely promoting the massive accumulation of anthocyanin only in Barbera (Fig. 3a).
562 In the leaf, *CHS3* may respond specifically to biotic stress. A pivotal role of CHS as key checkpoint
563 enzyme of flavonoid biosynthesis in response to pathogen infection has been reported previously in
564 many plant systems (Dao, Linthorst & Verpoorte 2011), however little is known about the specific

565 contribution of the single isoforms. Recently, it has been shown that within the *Glycine max* *CHS*
 566 family, *CHS4* did not accumulate, in contrast to the other isogenes, during response to
 567 *Pseudomonas syringae* pv. *glycinea*, thus suggesting gene-specific contributions to infection (Zabala
 568 *et al.* 2006). This hypothesis is supported by a study on grapevine leaves accumulating
 569 anthocyanins as a consequence of GLRaV-3 virus infection, which also showed a marked increase
 570 of *CHS3* expression and much smaller changes for *CHS1* and *CHS2* (Gutha *et al.* 2010).
 571 Comparing the two cultivars, beside differences in the magnitude of gene expression we also
 572 observed a cultivar-specific kinetic of *CHS3* induction, with the mean peak in August for Barbera
 573 and in July for Nebbiolo. A similar behaviour was observed for *F3H2*, suggesting a different timing
 574 in expression of “stem” biosynthetic genes between the two cultivars, that find a good
 575 correspondence with the total phenolics accumulation peak in Barbera and Nebbiolo (Fig. 2b).
 576 Interestingly, both upstream biosynthetic gene expression and phenolics accumulation trends, have
 577 correlated with that of phytoplasma titre (Fig. 2a), suggesting that flavonoid accumulation does not
 578 affect phytoplasma titre, while rather, is a consequence of FDp colonization, without excluding the
 579 influence of specific branch metabolites on the disease development.
 580 The three main specific groups of flavonoids in grapevine are anthocyanins, proanthocyanidins and
 581 flavonols. Fungal pathogens often activate proanthocyanidin biosynthesis (Kortekamp 2006;
 582 Polesani *et al.*, 2010; Rotter *et al.* 2009), while expression of genes controlling the anthocyanin
 583 branch, such as *UAGT* and *VvMYBA1* (Cutanda-Perez *et al.* 2009), and the flavonol branch, such as
 584 *FLS* and *VvMYBF1* (Czemmel *et al.* 2009), is not affected. Some grape viruses and phyoplasmas
 585 differently affect the expression of grapevine flavonoid biosynthetic genes and proteins, and,
 586 besides activating the proanthocyanidin branch, they markedly affect the anthocyanin branch
 587 inducing leaf reddening (Albertazzi *et al.* 2009; Guidoni *et al.* 1997; Gutha *et al.* 2010; Hren *et al.*
 588 2009a; Margaria *et al.* 2013). Viruses and phytoplasmas are retained in the symplast, lack cell walls
 589 and could partly escape sensing by the plant cells, thus not inducing the same responses observed in
 590 the case of fungal pathogens; many plant defence-related proteins in fact, have an antifungal or

glycan-lytic activity, that may limit the colonization of invading-microbes (van Loon, Rep & Pieterse 2006).

Anthocyanin concentration increased in both cultivars, but the accumulation was dramatic in Barbera, and much lower in Nebbiolo. Integrating metabolic and gene-expression data, we could find a significant correlation between *UAGT*, the anthocyanin-specific biosynthetic gene, and anthocyanin concentration in infected leaves. A mathematical model representing the relationship between these two variables (metabolite and gene) was found for each variety (Fig. 8). The model showed that the effect of *UAGT* accumulation on anthocyanins was double in Barbera (0.4556 Log_RQ versus 0.1761 Log_RQ) possibly suggesting genotype-dependent differences in *UFGT* efficiency. An explanation for the observed activation of anthocyanin biosynthesis may reside in the sugar concentration increase in infected leaves. High sugar level, in particular sucrose, is known to induce anthocyanin biosynthesis in *Arabidopsis* plants (Solfanelli *et al.* 2006) and in grapevine cell cultures (Gollop *et al.* 2002, Gollop, Farhi & Perl 2001). Accumulation of soluble carbohydrates and starch have been reported in phytoplasma-infected leaves of several hosts, such as periwinkle (Choi *et al.* 2004), coconut (Maust *et al.* 2003), papaya (Guthrie *et al.* 2001) and maize (Junqueira, Bedendo & Pascholati, 2004). In Bois Noir (BN) phytoplasma-infected grapevines cv Chardonnay, symptoms have been related to reduced photosynthetic activity and anomalous accumulation of carbohydrates (Bertamini & Nedunchezian 2001). Accordingly, gene expression studies showed significant changes in the grapevine carbohydrate transport and metabolism induced by BN phytoplasma (Albertazzi *et al.* 2009, Hren *et al.* 2009a, Santi *et al.* 2013). Phytoplasma infection in grapevine is therefore correlated to sugar increase, which is also related to callose accumulation, that cause physical obstruction of sieve tubes leading to inhibition of phloem loading and transport (Musetti 2010; Musetti *et al.* 2013).

An open question is whether anthocyanin and proanthocyanidin accumulation has any direct protective role toward the establishment of phytoplasma infection. The answer to this question is probably no, as flavonoids are accumulated in the wall and in vacuoles and thus spatially separated

617 from the phloem-restricted phytoplasmas. However, anthocyanin accumulation is important for
 618 protecting photosystems from oxygen radicals generated by photon-saturated photosystems in leaf
 619 cells. In fact under high light, low temperature, and chlorophyll loss, many plant leaves accumulate
 620 anthocyanins (Hoch, Singaas & McCown 2003). Decreasing photosynthetic rates and photosystem
 621 efficiency have been recently observed in leaves of plants displaying FD symptoms (Vitali *et al.*
 622 2013), and anthocyanins could have an antioxidant role, limiting further oxidative damage of leaf
 623 cells. However, anthocyanin accumulation could also act as a quencher of defence reactions
 624 mounted by the host plant. It has been demonstrated in fact, that peroxide radicals accumulates in
 625 the cell wall of phloem cells infected by the FD phytoplasma and it has been proposed that this
 626 accumulation can favour the recovery process (Gambino *et al.* 2013; Musetti *et al.* 2007). H₂O₂ can
 627 easily cross cell membranes and get in contact with the vacuoles containing anthocyanins, that are
 628 well-known antioxidants (Agati *et al.* 2012): consequently, anthocyanin accumulation could
 629 dampen the protective effect of oxygen radicals, resulting in increased phytoplasma growth. This
 630 observation is in line with the strong accumulation of anthocyanins and the higher phytoplasma titer
 631 found in Barbera. Our study showed that Nebbiolo leaves accumulate anthocyanins to a much lower
 632 extent than in Barbera (Fig. 3a), in agreement with our view of anthocyanin accumulation as a non-
 633 specific effect of FD-induced leaf sugar accumulation. Accordingly, this low anthocyanin
 634 concentration in Nebbiolo sustains a lower phytoplasma titer (Fig.2a).
 635 Proanthocyanidins concentration was generally higher in infected Barbera leaves (Fig. 3b). An
 636 interesting hallmark of Nebbiolo was the higher basal concentration of proanthocyanidins in healthy
 637 and recovered plants (Fig. 3b). This high constitutive proanthocyanidin concentration in the leaves,
 638 could explain Nebbiolo's low susceptibility to FD, possibly due to the repelling effect of these
 639 substances against feeding insects, as demonstrated in other plants against sucking pests (Mansour
 640 *et al.* 1997; Rao 2002).
 641 In agreement with the significant differences in the concentrations of proanthocyanidins in the two
 642 cultivars, we also found strong differences in the RQ of *LAR* transcripts, between Barbera and

643 Nebbiolo, and among the three sanitary states. We were able to fit the accumulation of PA with the
644 transcription levels of biosynthetic genes, and despite the strong differences between the varieties,
645 we found a single model correlating the three variables (Fig. 9).

646 Given the role of flavonoids as general modulators in stress responses, in particular hormone
647 balance (Pourcel *et al.* 2013), our findings provide a better understanding of the cross-talk between
648 different biochemical pathways and the chain reactions that can be induced by FDp infection, as it
649 was noticed earlier in proteomic studies (Margaria & Palmano 2011; Margaria *et al.* 2013). In
650 particular, the hormone jasmonic acid (JA) is known to be involved in regulating plant response to
651 stress; down-regulation of genes involved in its biosynthesis was observed in FDp- infected
652 grapevine leaves (Gambino *et al.* 2013). Interestingly, JA level was significantly reduced by
653 flavonoid accumulation in Arabidopsis (Pourcel *et al.* 2013). The flavonoid trend we have observed
654 in response to FDp infection did fit with these observations, suggesting that flavonoid increase
655 could have an effect on JA accumulation in grapevines as well. Moreover, extending the analysis to
656 other phenolic compounds other than flavonoids, would likely furnish new data on the complex
657 reaction of grapevine to phytoplasma infection: in this view, stilbenoids would be a good candidate
658 for future experimental works, given their documented role in other biotic interactions in grapevine
659 (Malacarne *et al.* 2011; Mattivi *et al.* 2011; Toffolatti *et al.* 2012; Vannozzi *et al.* 2013).

660 In conclusion, the results presented here, besides providing a molecular and biochemical description
661 of the flavonoid pathway in response to FDp infection in different grapevine cultivars, raise
662 intriguing hypothesis on its involvement in phytoplasma pathogenesis and on the response of
663 different grapevine genotypes to the disease. We tried our best to minimize the climatic and
664 agronomic differences between the different sampling sites, but of course, as we decided to study
665 the two cultivars in their original field-grown conditions, and due to the heterogeneity of vineyards,
666 we cannot exclude other stress factors that may have influenced the flavonoid pathway in the two
667 cultivars. However the similarity in light exposure, temperature, humidity and culture conditions
668 between the two sampling vineyards, together with the general biological behaviour of the two

669 cultivars in terms of symptom reactivity to FDp infection and supported phytoplasma titre, made us
670 confident that our speculations have a real confirmation in nature, and that the dramatic changes
671 observed between the two cultivars cannot be exclusively attributed to a restricted environmental
672 factor, but instead are a biological characteristic of the different grapevine genotypes in response to
673 phytoplasma infection.

674

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935 **Figure legends**

936 **Figure 1.** Different symptoms of Flavescence dorée infection on Barbera (a,b) and Nebbiolo (d,e)
937 leaves, and typical withering of grape berries (c).

938

939 **Figure 2.** Phytoplasma titer and total phenolic accumulation. a) Quantification of Flavescence dorée
940 phytoplasma in infected Barbera and Nebbiolo grapevines, reported as phytoplasma cells/ng leaf
941 DNA. b) Quantification of total phenolic concentration as equivalents of (+)-catechin hydrate in
942 healthy, FDp-infected and recovered leaves of Barbera and Nebbiolo during the 2011 vegetative
943 season.

944

945 **Figure 3.** Relative levels of: (a) total anthocyanin concentration (g kg⁻¹ as equivalents of malvidin
946 3-O-glucoside chloride); (b) total proanthocyanidin concentration (g kg⁻¹ as equivalents of (-)-
947 epicatechin gallate); (c) total flavonol concentration (as equivalents of quercetin 3-O-glucoside), in
948 the leaves of healthy, FDp-infected, and recovered plants of Barbera and Nebbiolo, at four sampling
949 dates expressed as days of the year (x axes). Means (three replicates \pm standard errors) were
950 separated by the Duncan's test (capital letters indicate significant differences for $P \leq 0.01$; lower
951 case letters for $P \leq 0.05$).

952

953 **Figure 4.** Expression of genes of the stem flavonoid pathway in healthy, Flavescence dorée
954 phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of
955 the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{-\Delta\Delta Ct_{weighted\ j,i}}$) were calculated for each
956 sample using the mean Ct value of all the healthy samples as reference. *CHS*: chalcone synthase;
957 *F3H*: flavanone-3-hydroxylase. Vertical bars represent standard errors.

958

959 **Figure 5.** Expression of genes of the anthocyanin branch in healthy, Flavescence dorée
960 phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of

the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{-\Delta\Delta Ct_{weighted\ j,i}}$) were calculated for each sample using the mean CT value of all the healthy samples as reference. *LDOX*: leucoanthocyanidin dioxygenase; *UAGT*: UGT-glucose:anohocyanin 3-Oglucosyltransferase; *VvMYBA1*: *UAGT*-transcription factor. Vertical bars represent standard errors.

Figure 6. Expression of genes of the proanthocyanidin branch in healthy, Flavescence dorée phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{-\Delta\Delta Ct_{weighted\ j,i}}$) were calculated for each sample using the mean CT value of all the healthy samples as reference. *ANR*: anthocyanidine reductase; *LAR*: leuco-anthocyanidine reductase. Vertical bars represent standard errors.

Figure 7. Expression of genes of the flavonol branch in healthy, Flavescence dorée phytoplasma- infected and recovered grapevine leaves, at four sampling dates expressed as days of the year (x axes). Relative quantities values ($RQ_{j,i} = 2^{-\Delta\Delta Ct_{weighted\ j,i}}$) were calculated for each sample using the mean CT value of all the healthy samples as reference. *FLS*: flavonol synthase; *VvMYBF1*: *FLS*-transcription factor. Vertical bars represent standard errors.

Figure 8. Relationship between Relative Quantity (RQ) of UGT-glucose:anohocyanin 3-Oglucosyltransferase (*UAGT*) gene and g/kg of anthocyanins (Log scale) and their regression lines. Infected plants of both cultivars showed a relationship between the anthocyanins concentration and transcript quantities, but the RQ in Barbera was significantly higher than in Nebbiolo and its effect on the anthocyanins concentration was almost double.

Figure 9. Representation of the relationship between proanthocyanidins (PA) accumulation and *LAR* and *ANR* relative quantities (RQ). (a) Fitted model representing the relationships of the concentration of proanthocyanidins [PA] with both the RQ of *LAR* and *ANR* transcripts for the two

987 cultivars together, without considering the sanitary states. (b) Distribution of the three sanitary
988 states separately for Barbera and Nebbiolo. Clustering of the infected samples toward the high
989 values of PA is evident in Barbera, in contrast to Nebbiolo, where the sanitary states are dispersed
990 along the levels of PA.

991

992

993 SUPPORTING INFORMATIONS

994 **Figure S1.** Experimental design of the study. The picture shows the complete work-flow, from
995 sampling in the field to biochemical and gene-expression analysis.

996

997 **Figure S2.** Climatic data obtained from meteorological stations for the two vineyards considered in
998 the study. (a) Mean Temperatures. (b) Growing degree days base 10 °C. (c) Relative humidity. The
999 red squares highlights the months considered for sampling.

1000

1001 **Table S1.** Reference source of the primers used in RealTime qRT-PCR reactions, for quantification
1002 of three reference genes, and ten candidate genes, together with GeneID, Genoscope gene
1003 accession, and primer amplification efficiencies in Barbera and Nebbiolo.

1004

1005 **Table S2.** Output values of the validation analysis of candidate reference genes, according to three
1006 software tools commonly used for reference gene selection: geNorm, Norm Finder, and Best
1007 Keeper.

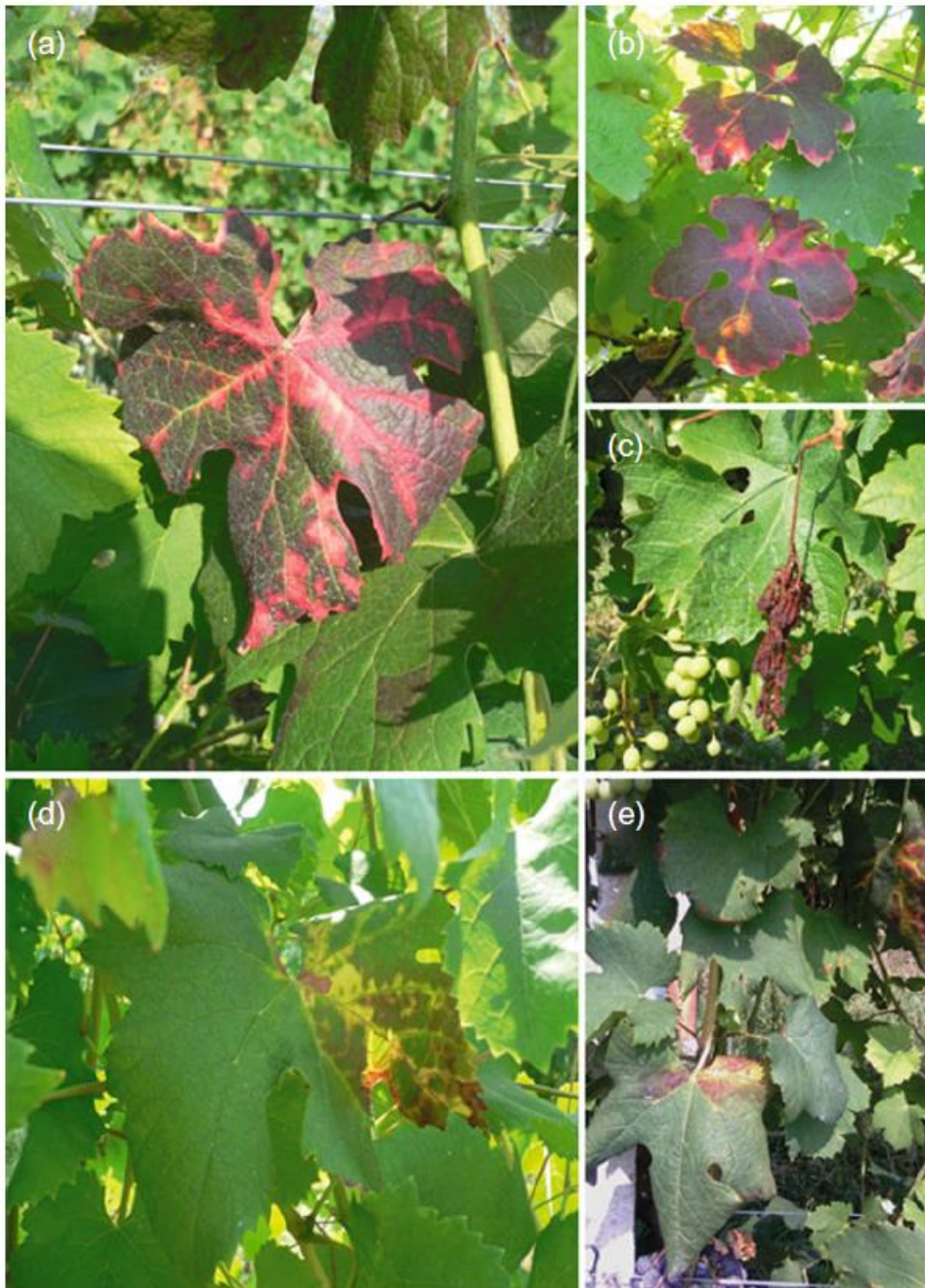
1008

1009 **Table S3.** Quantification of total anthocyanins by spectrophotometer analysis (absorbance read at
1010 520 nm), using malvidin 3-O-glucoside chloride as external standard reference. H: healthy sample;
1011 I: infected sample; R: recovered sample.

1012

1013 **Table S4.** Quantification of total proanthocyanidins as equivalents of (-)-epicatechin gallate by
 1014 spectrophotometer analysis. H: healthy sample; I: infected sample; R: recovered sample.
 1015

1016 **Table S5.** Quantification of flavonols by HPLC-DAD, in Barbera (A) and Nebbiolo (B) healthy,
 1017 Flavescente dorée phytoplasma-infected and recovered leaves.
 1018 Means of three replicates \pm standard errors; TF = total flavonol (g/kg), Myr mG (Myricetin 3-O-
 1019 glucoside), Q glr (quercetin 3-O-glucuronide), Q mG (quercetin 3-O-glucoside), K glr (kaempferol
 1020 3-O-glucuronide), K mG (kaempferol 3-O-glucoside). M = percentage of myricetin 3-O-glucoside
 1021 over total flavonols; Q = percentage of quercetins over total flavonols; K = percentage of
 1022 kaempferols over total flavonols.
 1023
 1024
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1026

1027 Figure 1

1028

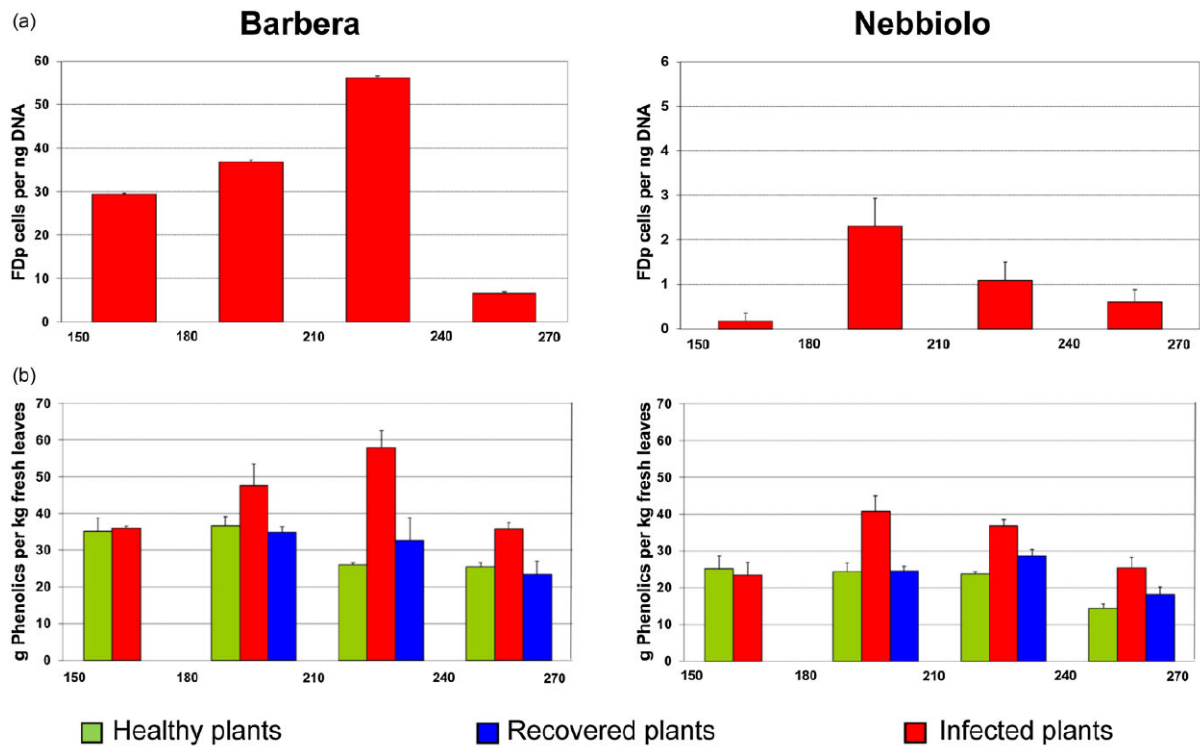
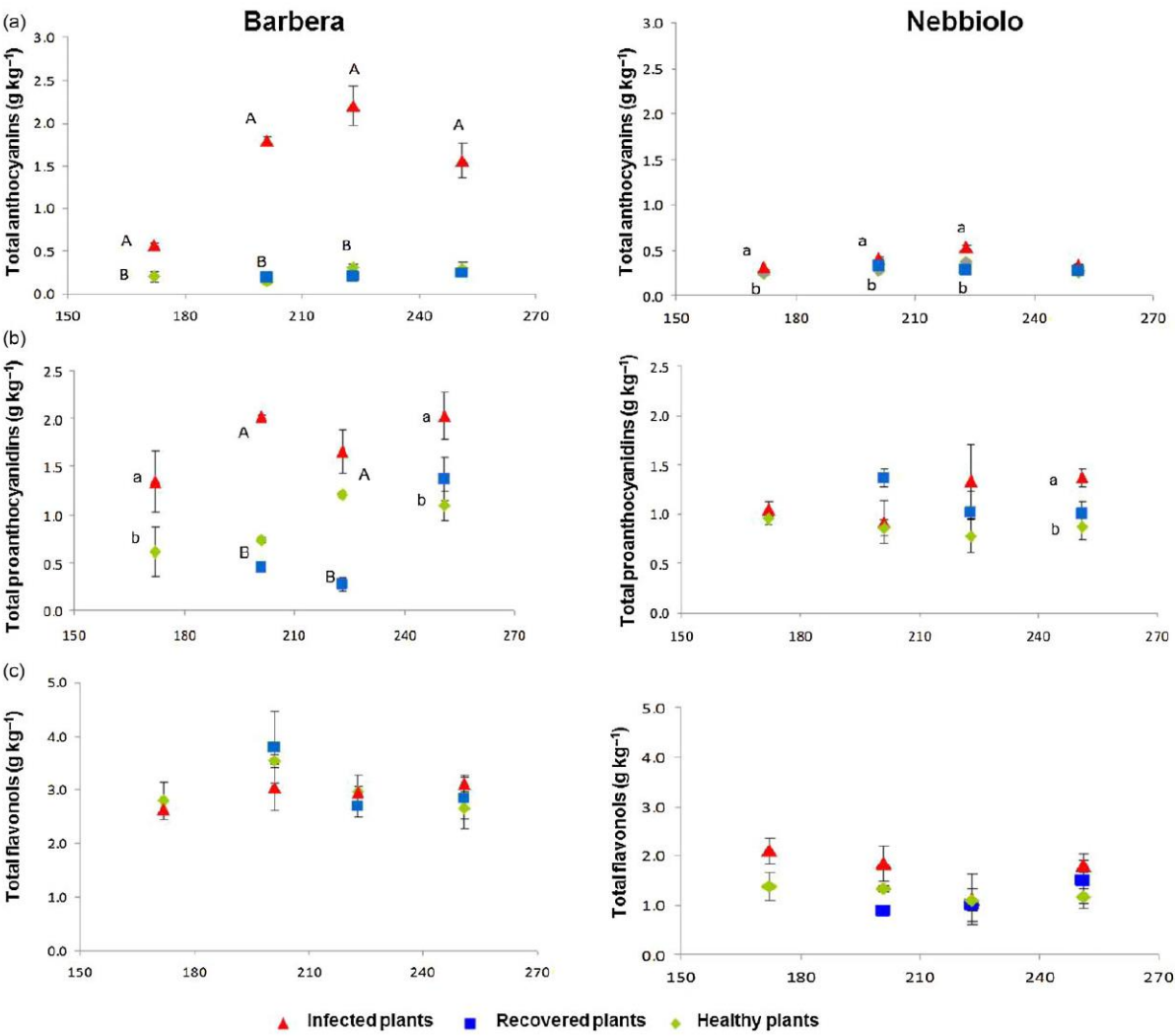


Figure 2

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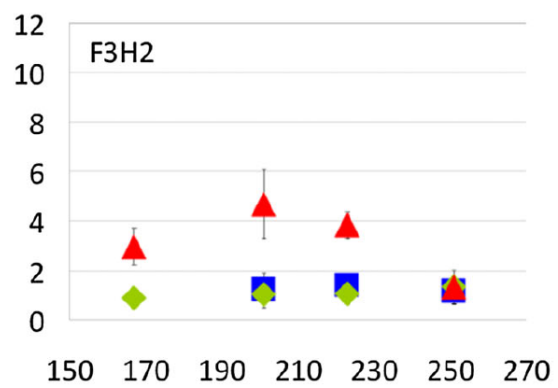
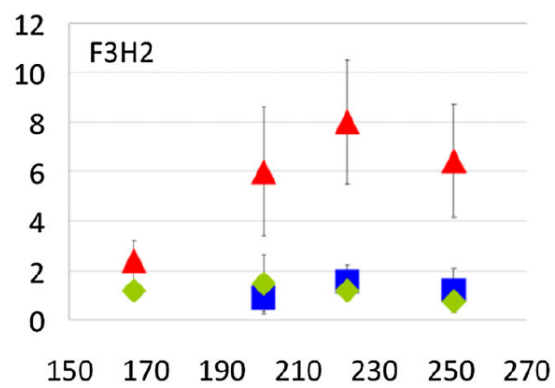
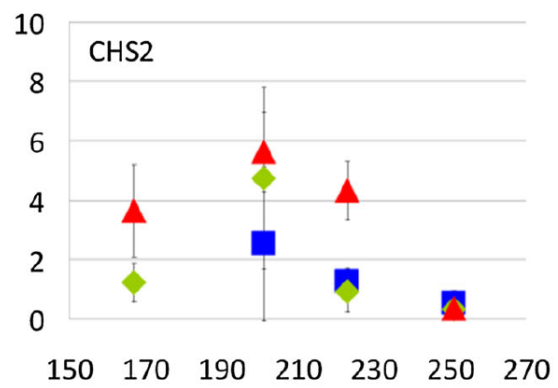
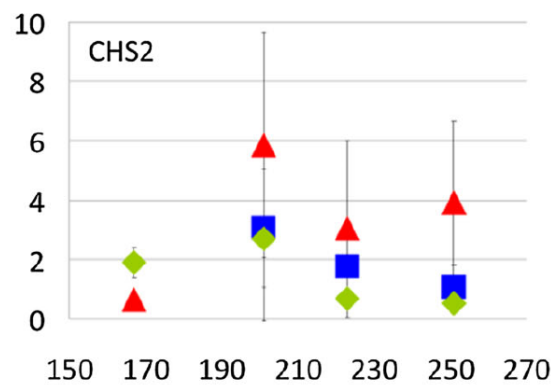
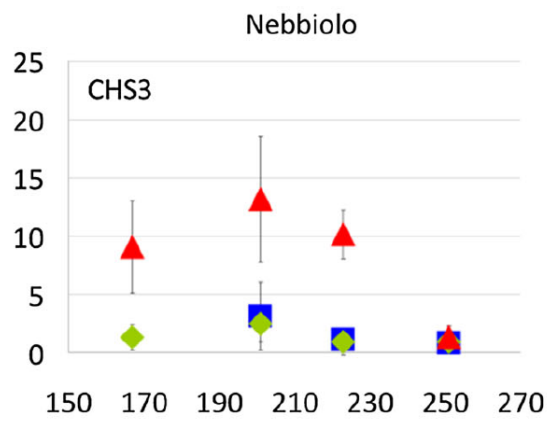
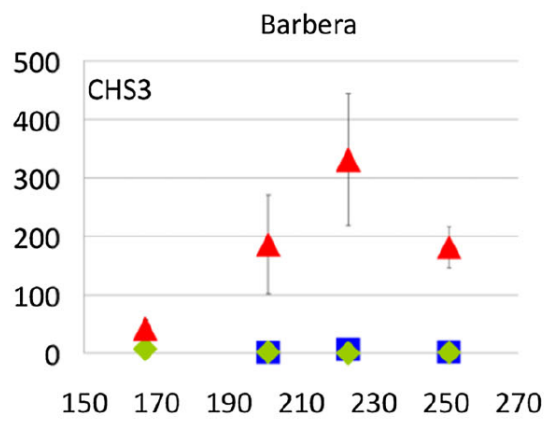


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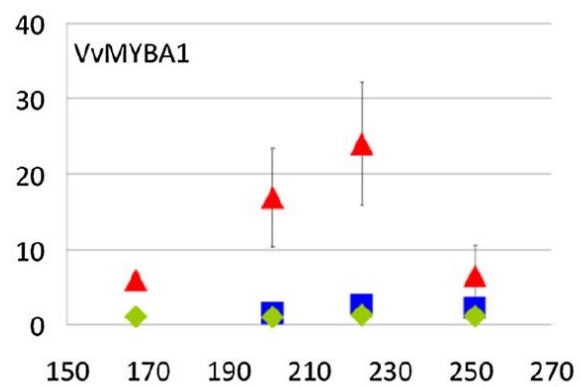
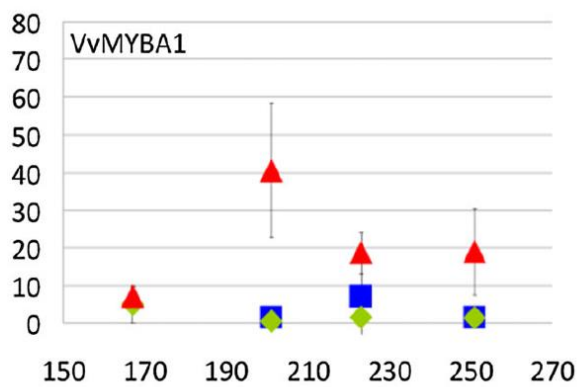
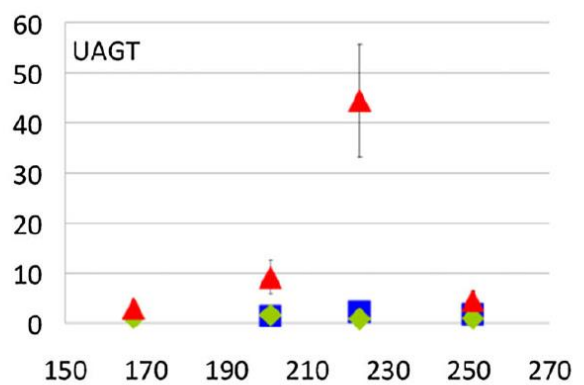
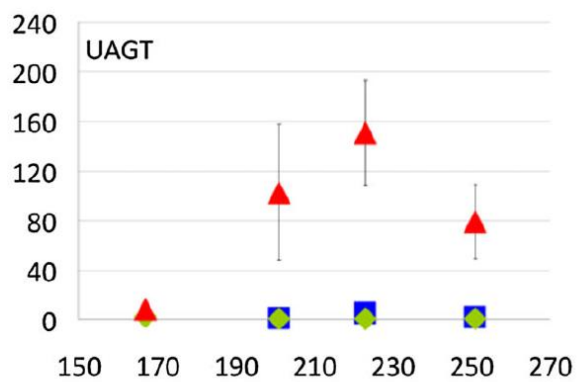
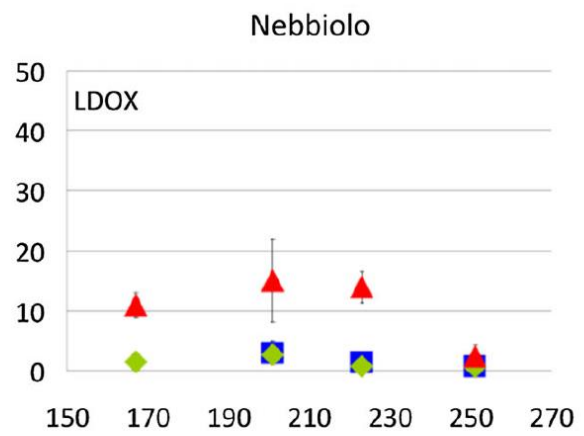
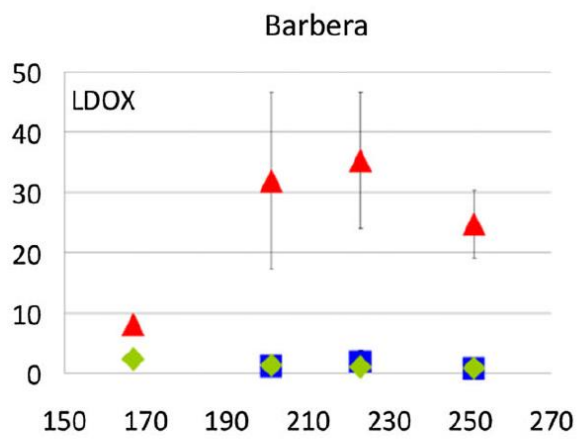
1036 Figure 3

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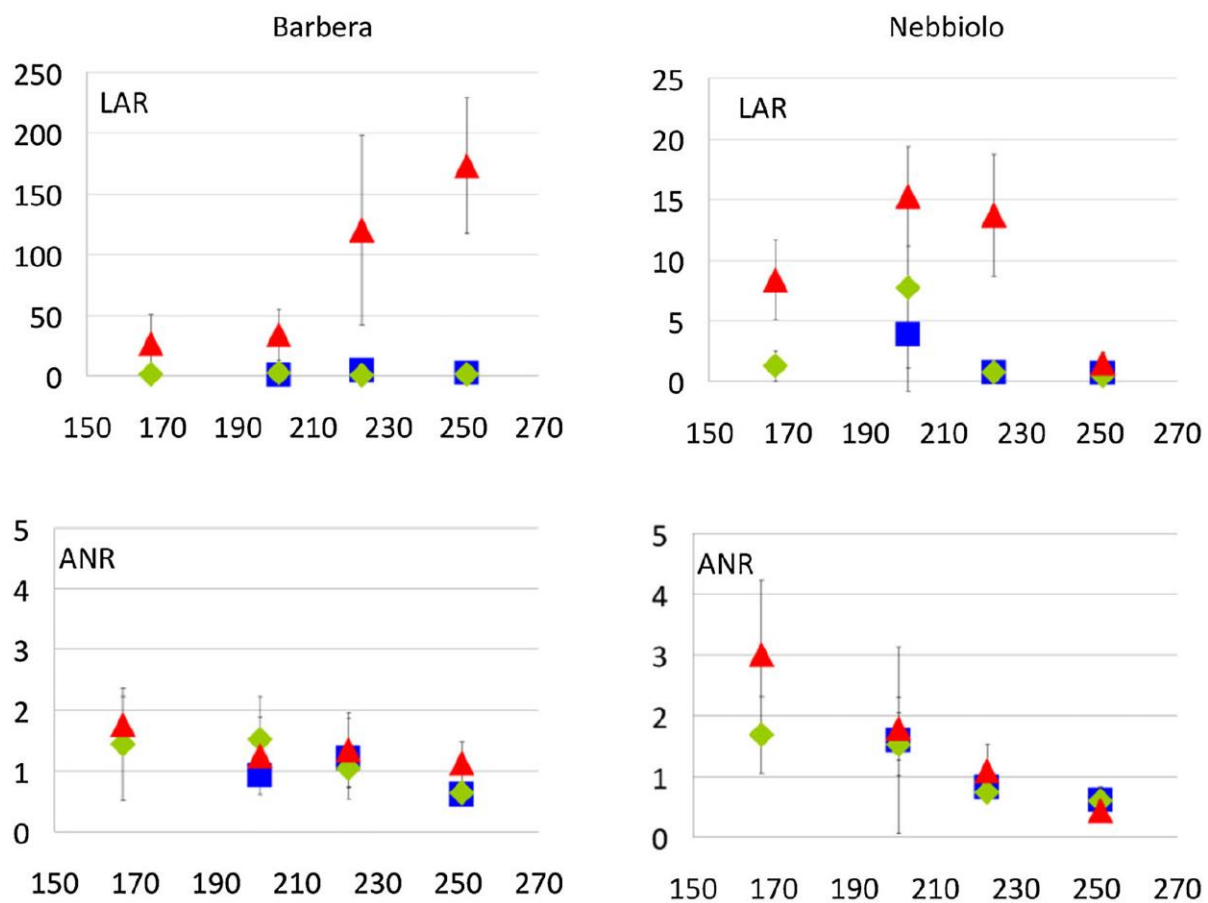
▲ Infected plants
 ■ Recovered plants
 ◆ Healthy plants

Figure 4



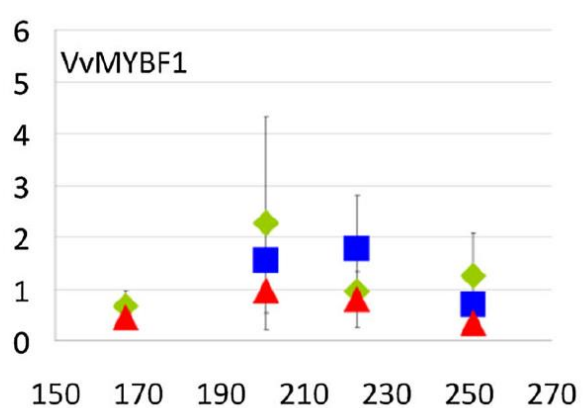
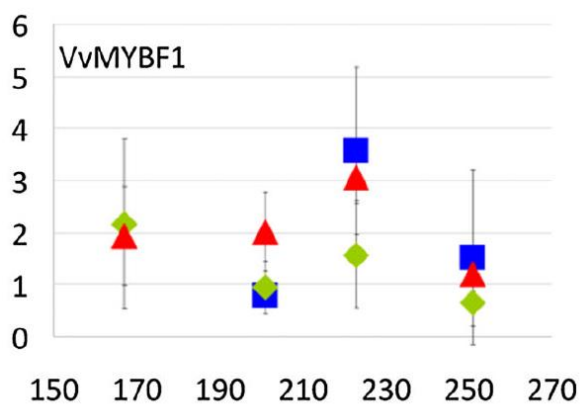
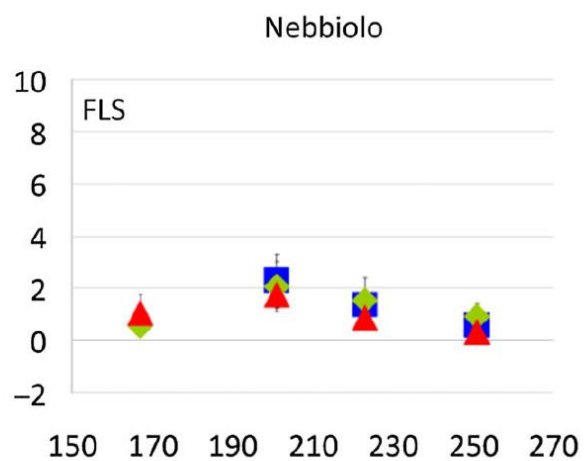
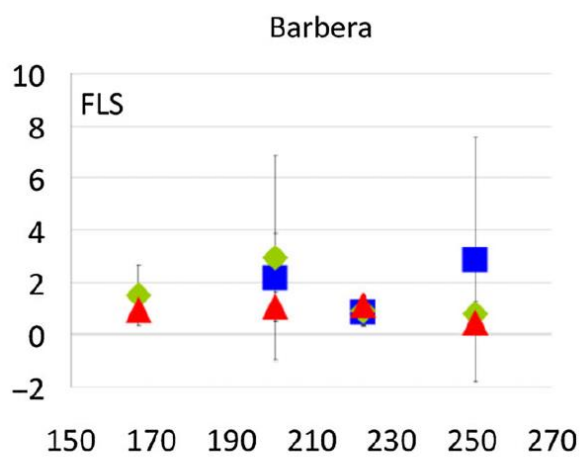
▲ Infected plants ■ Recovered plants ◆ Healthy plants

Figure 5



▲ Infected plants
 ■ Recovered plants
 ◆ Healthy plants

Figure 6



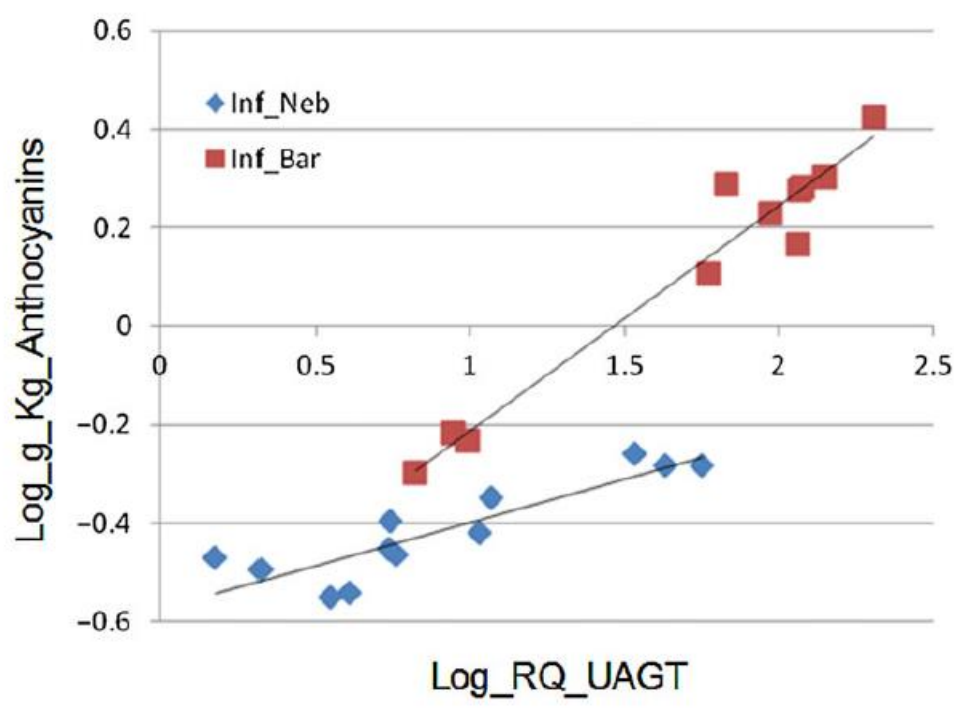
▲ Infected plants

■ Recovered plants

◆ Healthy plants

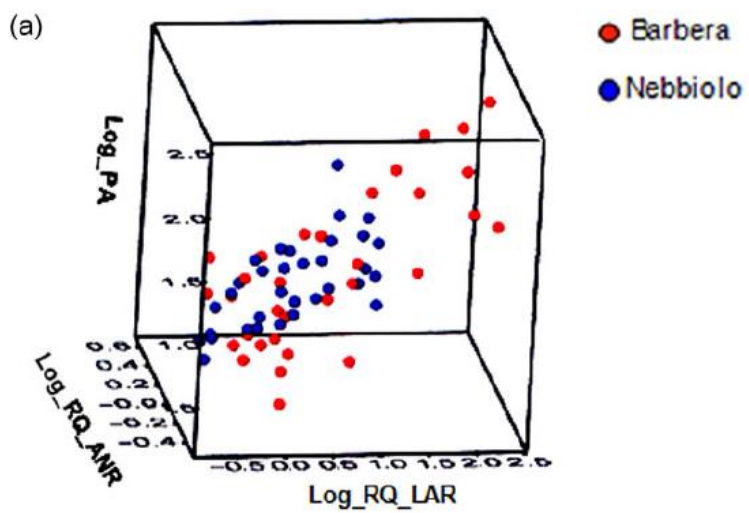
Figure 7

1050

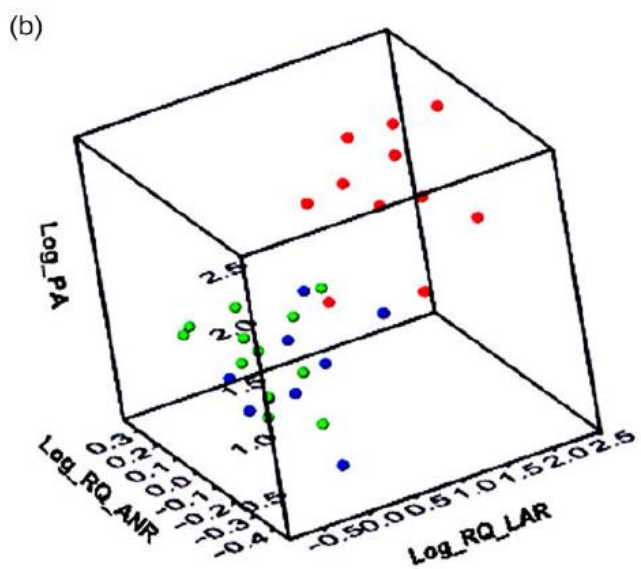


1051

1052 Figure 8



Barbera



Nebbiolo

